

Applicant : John Bertin
Serial No. : 09/728,721
Filed : December 1, 2000
Page : 7 of 11

Attorney's Docket No.: 07334-315001 / MPI97-045CP5

REMARKS

Claims 23-28, 30, 31, and 33-56 are pending in the application. Claims 29 and 32 have been canceled without prejudice. Claims 28, 30, 31, and 33 have been amended to incorporate the limitations of the cancelled claims and modify claim dependencies. These amendments add no new matter.

Allowable Subject Matter

At page 7 of the Office Action, the Examiner stated that claims 23-27 and 38-51 are allowed and that claims 29, 30, 32, 33, 53, and 54 are objected to as being dependent upon rejected base claims, but would be allowable if rewritten in independent form to include all of the limitations of the base claim and any intervening claims. In light of the claim amendments and comments provided herein, applicant respectfully submits that all claims are now in condition for allowance.

35 U.S.C. §112, First Paragraph (Written Description)

At pages 2-7 of the Office Action, the Examiner rejected claims 28, 31, 34-37, 52, 55, and 56 as allegedly containing subject matter that was not described in the specification in such a way that one skilled in the art can reasonably conclude that applicant had possession of the claimed invention at the time of filing.

Claims 28, 30, 31, and 33 have been amended and claims 29 and 32 have been cancelled. For the reasons provided below, applicant respectfully submits that all of the pending claims meet the written description requirement.

(i) Percent Identity

Independent claims 28 and 31 have each been amended to incorporate all of the limitations of allowable claims 29 and 32, respectively. In light of these amendments, claims 29 and 32 have been cancelled. As amended, claims 28 and 31 require that the claimed nucleic acid contain a nucleotide sequence that encodes a polypeptide containing an amino acid sequence that

Applicant : John Bertin
Serial No. : 09/728,721
Filed : December 1, 2000
Page : 8 of 11

Attorney's Docket No.: 07334-315001 / MPI97-045CP5

is at least 95% identical to the sequence of SEQ ID NO:49. In light of these amendments, applicant requests that the Examiner withdraw the rejections of claims 28 and 31.

(ii) Hybridization

Claims 34-37, 52, 55, and 56 are drawn to a nucleic acid containing a nucleotide sequence that: (a) hybridizes to a reference polynucleotide sequence under defined conditions of hybridization and washing; and (b) encodes a polypeptide that stimulates NF-kB activity (claims 34, 35, 52, and 55) or binds to caspase-1 (claims 36, 37, 52, and 56).

At pages 6-7 of the Office Action, the Examiner stated that

Applicant argues that claims 34-37, 52, 55, and 56 have adequate written description since the hybridization conditions recited in the claims provide for the hybridization of only structurally similar nucleic acids. It is noted that at page 39 of the specification the hybridization conditions recited in the claims are conditions that are "stringent" and the specification at this page provides that these conditions will allow that the nucleic acids in the claims are only at least 60%-70% identical to SEQ ID NO:50 or the cDNA in ATCC PTA-213. The hybridization language provides no coupling of a structure correlated with the functions characterized in the specification

Claims 34-37, 52, 55, and 56 require that the claimed nucleic acid contain a nucleotide sequence that hybridizes to a recited polynucleotide sequence under conditions of (i) hybridization at 45°C in 6.0 X sodium chloride/sodium citrate (SSC), and (ii) washing in 0.2 X SSC, 0.1% sodium dodecyl sulfate (SDS) at 65°C. The washing conditions recited in these claims are highly stringent due to the requirement of a low salt concentration (0.2 X SSC) combined with high temperature (65°C). As a result of these high stringency washing conditions, only nucleic acids highly related to the recited polynucleotide sequence will remain hybridized following the hybridization and washing steps specified in the claims. In particular, the high stringency washing conditions will cause the washing away of significantly mismatched nucleic acids that may initially hybridize to the target polynucleotide during the hybridization step that precedes the wash. Enclosed as "Exhibit A" is Section 6.3 of Current Protocols of Molecular Biology, which describes a "high stringency wash buffer" having 0.2 X SSC and

Applicant : John Bertin
Serial No. : 09/728,721
Filed : December 1, 2000
Page : 9 of 11

Attorney's Docket No.: 07334-315001 / MPI97-045CP5

0.1% SDS (page 6.3.4) and the use of such a buffer at high washing temperatures to detect hybridization between highly related probe and target sequences (page 6.3.6).

The specification states that the phrase "hybridizes under stringent conditions" is intended to encompass conditions for hybridization and washing under which nucleotide sequences at least 60-75% identical remain hybridized to each other (specification at page 39, lines 19-26; emphasis added). This passage and its use of the words "at least" does not exclude that certain stringent conditions can result in the hybridization of only nucleic acids having higher levels of percent identity. Consistent with these comments, currently pending claims 34-37, 52, 55, and 56 were drafted to require that a very high stringency wash (low salt and high temperature) be carried out, thus requiring a degree of relatedness between the two sequences that is higher than the minimum levels recited at page 39 of the specification.

The genus of nucleic acids encompassed by claims 34-37, 52, 55, and 56 does not have substantial variation, since each nucleic acid must encode a polypeptide that has a specified activity (i.e., the ability to stimulate NF-kB activity or bind caspase-1) and contain a structurally similar nucleotide sequence (i.e., one that hybridizes under the hybridization and washing conditions recited in the claims). The CARD-5 nucleic acids disclosed in the specification are representative of the claimed genus because: all members of the genus hybridize under high stringency to a CARD-5 nucleic acid; and the specification describes assays for identifying variants encompassed by the claim having the specified activity (such assays are described in the specification at, e.g., page 128, lines 10-26, and page 118, line 15, to page 120, line 5). In light of this disclosure, the skilled artisan would have concluded, at the filing of the present application, that applicant was in possession of the necessary common attributes possessed by the members of the genus.

The Examiner cited *Regents of the University of California v. Lilly & Co.*, 119 F.3d 1559 (Fed. Cir. 1997) in support of the present rejection. The discussion in *Lilly* regarding a proper written description for genus claims had to do with a claim drawn to a vertebrate mRNA encoding insulin. The *Lilly* court held that a generic statement, such as the term "mammalian insulin cDNA" is not, without more, an adequate written description of an invention claiming the

Applicant : John Bertin
Serial No: 09/728,721
Filed : December 1, 2000
Page : 10 of 11

Attorney's Docket No.: 07334-315001 / MPI97-045CP5

nucleotide sequence for human insulin. The court's decision in *Lilly* focused on functional claims directed merely to a desired result without structure: "[t]he description requirement of the patent statute requires a description of an invention, not an indication of a result that one might achieve if one made that invention." *Id.* at 1406. However, the *Lilly* court also took care to indicate that structural information about the claimed genus was different in kind than a mere desired result. The court indicated that in claims involving chemical materials such as proteins and polynucleotides "generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is usually an adequate description of the claimed genus." *Id.*

The present claims are drawn to nucleic acids identified, in part, by their ability to hybridize to a reference polynucleotide sequence under a set of defined hybridization and washing conditions. The ability of a nucleic acid molecule to hybridize to a reference nucleic acid molecule under such defined conditions is dependent on the structure (sequence) of the nucleic acid molecule. Moreover, the claimed nucleic acids are also defined by the recited function of the polypeptide encoded by the nucleotide sequence (i.e., the ability to stimulate NF-kB activity (claims 34, 35, 52, and 55) or the ability to bind caspase-1 (claims 36, 37, 52, and 56)). The claims are not directed to a desired result without structure, as was the case in *Lilly*. A person of ordinary skill in the art would clearly understand the structural definition of the nucleic acids provided by the claims and would therefore understand applicant to have been in possession of the claimed nucleic acids at the time the application was filed. Accordingly, applicant respectfully submits that the pending "hybridization" claims satisfy the written description requirement.

Applicant : John Bertin
Serial No. : 09/728,721
Filed : December 1, 2000
Page : 11 of 11

Attorney's Docket No.: 07334-315001 / MPI97-045CP5

Conclusions

Applicant asks that all claims be allowed in view of the amendments to the claims and the remarks presented herein.

Enclosed is a Notice of Appeal and a Petition for Two Month Extension of Time. Please apply the \$420 Petition for Extension of Time fee and the \$330 Notice of Appeal fee (and any other charges or credits) to deposit account 06-1050, referencing Attorney Docket No. 07334-315001.

Respectfully submitted,

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EXHIBIT A

SECTION II

HYBRIDIZATION WITH
RADIOACTIVE PROBES

After plaques or colonies have been transferred to nitrocellulose filters, the desired clone can be detected by its ability to hybridize to a DNA probe. This is a rapid, effective screening procedure that allows the identification of a single clone within a population of millions of other clones. The filters are hybridized with a ^{32}P -labeled nucleic acid probe, the excess and incorrectly matched probe is washed off the filter, and the filter is autoradiographed. Two features of the nucleic acid probe used for these experiments are critical to the successful screening of recombinant DNA libraries. First, the probe must hybridize only to the desired clones and not to any other clones. Thus, the nucleic acid sequence used for a probe must not contain any reiterated sequences or sequences that will hybridize to the vector. Second, the specific activity of the probe must be at least 10^7 cpm/ μg . Most of the procedures for labeling DNA or copy RNA molecules are described in Chapter 3, and a support protocol is presented here that allows the 5' end-labeling of a mixture of oligonucleotides.

The two basic protocols presented in this section describe steps required to hybridize labeled probes to recombinant DNA clones on filters. Two protocols are presented because conditions for hybridizing short oligonucleotide probes and longer nucleic acid probes to filters are different.

UNIT 6.3

BASIC
PROTOCOL

Using DNA Fragments as Probes

HYBRIDIZATION IN FORMAMIDE

Bacteriophage plaques or bacterial colonies bound to a filter membrane are detected by hybridization with a radioactive probe. Hybridization proceeds on prewet filters placed in a sealable plastic bag. After hybridization the filters are removed from the sealed bag, excess probe is washed off, and the filters are autoradiographed to identify the clones that have hybridized with the probe.

Materials

Nitrocellulose membrane filters bearing plaques, colonies, or DNA (UNITS 6.1 & 6.2)
Hybridization solution I
Radiolabeled probe, 1 to 15 ng/ml (UNIT 3.5)
2 mg/ml sonicated herring sperm DNA
High-stringency wash buffer I
Low-stringency wash buffer I
Sealable bags
42°C incubator
Water bath adjusted to washing temperature (see commentary)
Glass baking dish
Additional reagents and equipment for autoradiography (APPENDIX 3)

Incubate filters with probe

1. Wet filters with hybridization solution I. Lay a filter membrane bearing plaques on top of 5 to 20 ml of hybridization solution I and allow solution to seep through filter. It is important to wet only one surface at a time to prevent trapping air in filter. Wet each filter in turn, producing a stack of wet filters.

When multiple filters are to be hybridized to the same probe, no more than twenty 8.2-cm discs or ten 20 × 20 cm square filters should be placed in one stack.

Using DNA
Fragments
as Probes

6.3.1

Supplement 24 CFMB

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Estimate the volume of hybridization solution used to wet the filters; this is a significant fraction of the volume of the hybridization reaction.

2. Transfer the stack of wetted filters to an appropriately sized sealable bag. Add enough hybridization solution to generously cover filters and seal.

Note the volume of hybridization solution used to cover the filters.

3. Prehybridize filters by placing the bag in a 42°C incubator for at least 1 hr.

Some investigators omit this step.

4. While filters are prehybridizing, pipet the radioactive probe into a screw-cap tube, add 2 mg (1 ml) sonicated herring sperm DNA, and boil 10 min. Place boiled probes directly into ice to cool.

The amount of probe used is important, and should be in the range of 1 to 15 ng/ml of hybridization reaction. The volume of the hybridization reaction can be assumed to be the amount of hybridization solution added to the filters.

5. Add 2 ml hybridization solution I to the boiled probe.
6. Remove bag containing filters from the 42°C incubator. Open bag, add probe mixture, exclude as many bubbles as possible, and reseal.

A good way to add the radioactive probe is to take it up in a syringe with an 18-G needle and then inject it into the bag. Reseal the bag after adding probe.

7. Mix probe in the bag so that filter is evenly covered. Replace bag in the 42°C incubator and let hybridize overnight.

Wash filters to remove nonhybridized probe

8. Warm 1 liter high-stringency wash buffer I to the "washing temperature" in a water bath.

The stability of washing temperature and salt concentrations are critical features of this experiment. See discussion in commentary.

9. Remove bag containing hybridizing filters from the 42°C incubator. Cut bag open and squeeze hybridization solution out of the bag.

CAUTION: *Handle material carefully as it is extremely radioactive. This should be done on disposable paper bench covers.*

10. Quickly immerse the filters in 500 ml low-stringency wash buffer I at room temperature in a glass baking dish. Separate all the filters, as they may stick together during hybridization.

The volume of the low-stringency wash buffer is not important as long as the filters are completely covered. The filters must not be allowed to dry as the radioactive probe will irreversibly bind the filters if the filters dry in contact with probe. (The type of container used to hold the filters is not important as long as it transfers heat well. Thus glass, metal, or enamel containers are better than plastic.)

The low-stringency wash only removes nonhybridized probe formamide and hybridization solution; it does not determine the stringency of the hybridization.

11. Rinse the filters three times with 500 ml low-stringency wash buffer. Let the filters sit 10 to 15 min at room temperature in low-stringency wash buffer with each rinse.
12. Pour off the low-stringency wash buffer and pour in 500 ml high-stringency wash buffer (prewarmed to washing temperature).

Screening
Recombinant
DNA Libraries

6.3.2

13. Replace the high-stringency wash buffer with another 500 ml of high-stringency wash buffer, then place the glass dish containing the filters in incubator at wash temperature. Make sure that the temperature in the glass dish reaches the desired washing temperature by placing a thermometer directly into the bath and measuring the temperature. Usually 15 to 20 min at the desired wash temperature is sufficient to remove most of the background radioactivity.

Of course, if the glass dish is placed in a water bath, be careful that the water from the water bath does not get into the filters.

Autoradiographing filters

14. Remove filters and mount them either wet or dry on a plastic backing. If the filter(s) is to be exposed wet, then isolate it from the film by covering it with plastic wrap.

Used X-ray film provides a good form of plastic backing for filters.

15. Mark the filters with radioactive ink to assist in alignment and autoradiograph.

An easy way to apply radioactive ink is to mark adhesive-backed paper labels with radioactive ink and then attach the stickers to the plastic wrap cover.

X-ray intensifying screens greatly decrease the amount of exposure time required.

ALTERNATE PROTOCOL

HYBRIDIZATION IN AQUEOUS SOLUTION

This method differs mainly in that formamide is not used in the hybridization solution. Follow the basic protocol except use the reagents and alternate parameters listed below.

Additional Materials

Hybridization solution II
Low-stringency wash buffer II
High-stringency wash buffer II
65°C incubator

1. Prehybridize as in basic protocol except that the filters are prehybridized at 65°C using hybridization solution II.

Hybridization solution II may have to be prewarmed to solubilize the SDS.

2. Prepare probe as in step 4 of basic protocol and dilute with 2 ml of hybridization solution II.
3. Hybridize overnight as in steps 6 and 7 of basic protocol except use a hybridization temperature of 65°C.
4. Remove bag containing hybridization from the 65°C incubator. Squeeze out the hybridization solution, taking care to avoid contamination with the excess radioactive hybridization solution.
5. Immediately rinse filters twice with low-stringency wash buffer II.

It is unnecessary to maintain a given temperature for this wash; just let the filters sit in wash buffer at room temperature until ready to proceed.

6. At 65°C, proceed to wash filters with high-stringency wash buffer II. Employ multiple quick washes (5 to 8) and immerse filter in a final wash for ~20 min. Check the radioactivity of the filters with a Geiger counter and be certain that they produce a signal only a fewfold above background levels.

Using DNA
Fragments
as Probes

6.3.3

Current Protocols in Molecular Biology

REAGENTS AND SOLUTIONS**High-stringency wash buffer I**

0.2× SSC (APPENDIX 2)

0.1% sodium dodecyl sulfate (SDS)

High-stringency wash buffer II1 mM Na₂EDTA40 mM NaHPO₄, pH 7.2

1% SDS

Hybridization solution I

Mix following ingredients for range of volumes indicated (in milliliters):

Formamide	24	48	72	120	240	480
20× SSC	12	24	36	60	120	240
2 M Tris-Cl, pH 7.6	0.5	1.0	1.5	2.5	5.0	10
100× Denhardt's solution	0.5	1.0	1.5	2.5	5.0	10
Deionized H ₂ O	2.5	5.0	7.5	12.5	25	50
50% dextran sulfate	10	20	30	50	100	200
10% SDS*	0.5	1	1.5	2.5	5	10
Total volume	50	100	150	250	500	1000

*In place of SDS, *N*-laurylsarcosine (Sarkosyl) may be used.

Add the SDS last. The solution may be stored for prolonged periods at room temperature.

The dextran sulfate should be of high quality. Pharmacia produces acceptable-grade dextran sulfate. Recipes for SSC and Denhardt's solution are in APPENDIX 2.

Hybridization solution II

1% crystalline BSA (fraction V)

1 mM EDTA

0.5 M NaHPO₄, pH 7.2 (134 g Na₂HPO₄·7H₂O plus 4 ml 85% H₃PO₄/liter = 1 M NaHPO₄)

7% SDS

Low-stringency wash buffer I

2× SSC (APPENDIX 2)

0.1% SDS

Low-stringency wash buffer II

0.5% BSA (fraction V)

1 mM Na₂EDTA40 mM NaHPO₄, pH 7.2

5% SDS

Sonicated herring sperm DNA, 2 mg/ml

Resuspend 1 g herring sperm DNA (Boehringer Mannheim #223636) in a convenient volume (about 50 ml of water) by sonicating briefly. The DNA is now ready to be sheared into short molecules by sonication. Place the tube containing the herring sperm DNA solution in an ice bath (the tube must be stable even if the ice begins to melt). The sonicator probe is placed in the DNA solution (without touching the bottom of the vessel). The sonicator is turned on to 50% power 20 min, or until there is a uniform and obvious decrease in viscosity. At no time should the tube containing the DNA become hot to the touch. After sonication, the DNA is diluted

Screening
Recombinant
DNA Libraries

6.3.4

Supplement 13

Current Protocols in Molecular Biology

to a final concentration of 2 mg/ml, frozen in 50-ml aliquots, and thawed as needed.

COMMENTARY

Background Information

All hybridization methods depend upon the ability of denatured DNA to reanneal when complementary strands are present in an environment near but below their T_m (melting temperature). In a hybridization reaction involving double-stranded DNA on a filter and a single-stranded DNA probe there are three different annealing reactions occurring. First, there are the desired probe-DNA interactions which result in signal. Second, there are mismatch interactions that occur between related but non-homologous sequences; these mismatch hybrids are the ones that must be eliminated during the washing of the filters. Non-sequence-specific interactions also occur and these result in noise. The ability to extract information from a particular hybridization experiment is a function of the signal-to-noise ratio. High background or poor specific signal can both result in uninterpretable results.

Washing nitrocellulose filters is required to remove excess radioactive probe, as well as radioactive probe that has bound to the DNA on the filter as mismatch hybrids. Temperature and salt concentration dramatically affect the maintenance of specific hybrids. Detergents and other charged species can have a profound effect upon the nonspecific binding of species that contribute to background. In this protocol, hybridization is achieved in a solution containing 50% formamide. Excess probe is rinsed away under low-stringency conditions so that further hybridization will not occur. Once the hybridization solution is rinsed away, it is possible to proceed to a high-stringency wash without fear of further hybridization. When washing is complete, the filters should produce very little "noise" when monitored with a Geiger counter. Although single-copy sequence probe normally does not produce a signal that is detectable with a Geiger counter, a probe corresponding to more abundant sequences will produce a signal that can be "heard" with a Geiger counter.

Literature Review

Hybridization to filter membranes forms a basis of recombinant DNA technology and is described in detail earlier in the manual (*UNIT 2.9*). The protocols described here vary from those used for Southern blot filter hybridization in that the volume of the hybridization is usually larger and the washing conditions are different. Dextran sulfate is an important component of the hybrid-

ization solution as it increases the rate of reassociation of the nucleic acids.

The protocols in this unit describe methods for hybridizing radioactive probes to membrane-bound plaques or colonies. These procedures for screening recombinant clones were first suggested by Grunstein and Hognes (1975) and by Benton and Davis (1977). The conditions of hybridization proposed in the basic protocol involving hybridization in formamide was originally described by Denhardt (1966) and Gillespie and Spiegelman (1965) while the alternate protocol using aqueous hybridization solution was introduced by Church and Gilbert (1984).

The method of washing filters under stringent conditions to remove background was first proposed by Southern (1975). Botchan et al. (1976) described the benefit of adding SDS to the wash solution. Jeffreys and Flavell (1977) first employed the wash conditions described in the protocols presented here.

Critical Parameters

Hybridization. Kinetically, the hybridization of DNA (or RNA) probes to filter-bound DNA is not significantly different from hybridization in solution. For single-stranded probes, the rate of hybridization follows first-order kinetics, since probe is available in excess. Under conditions of excess probe, the time for hybridization is inversely proportional to the probe concentration. For double-stranded probes the rate of hybridization displays a more complex relationship to the initial probe concentration. However, to a first approximation the initial probe concentration is inversely proportional to the rate of hybridization. To determine the actual time required for the successful hybridization of a given probe, either empirical data must be available or the following formula can be used to determine the length of time (in hours) required to achieve 50% hybridization (T_{50}):

$$\frac{1}{2} \times \frac{1}{x} \times \frac{1}{y} \times 2 = T_{50}$$

where x is the weight of probe in micrograms; y is the complexity of probe in kilobases; and z is the volume of hybridization solution in milliliters. The length of time T is given in hours. Maximum hybridization signal will be obtained if the reaction is allowed to proceed to $5 \times T_{50}$, although 1 to $2 \times T_{50}$ is often used.

It is also clear that nonspecific interactions

Using DNA
Fragments
as Probes

6.3.5

Supplement 13

Current Protocols in Molecular Biology

occur and that in any hybridization, sources of noise will be present. Therefore, from a practical standpoint one conventionally utilizes concentrations of nick-translated probe on the order of 1 to 15 ng/ml of hybridization, where the specific activity of the probe is from 5×10^7 cpm/ μ g to $>10^8$ cpm/ μ g. Too much probe in a hybridization is as bad as too little.

One important source of background hybridization to filters is due to the hybridization of the probe to vector sequences or to *E. coli* DNA. Be certain that there is no vector or *E. coli* DNA sequences in the probe. This can best be ensured by isolating the probe from one type of vector (e.g., plasmid) and screening a library made with a different type of vector (e.g., bacteriophage).

Washing temperature. Washing at low stringency is a straightforward proposition. Buffer is added at room temperature and washing proceeds at room temperature.

High-stringency wash is determined empirically. The relative homology between the probe and target sequence is a determining parameter. If the homology is 100%, a high temperature (65° to 75°C) can be used. As the homology drops, lower washing temperatures must be used. In general one starts at 37° to 40°C, raising the temperature by 3° to 5°C intervals until background is low enough not to be a major factor in the autoradiography.

The length of the probe is also important. Very short probes (<100 bp) must be washed at lower temperatures, even if the homology is 100%. Washing strategy is the same as for probes of differing homology.

Salt concentration. The lower the salt concentration, the higher the stringency. With this said, the protocols as outlined do not require adjustment of salt concentration for adjustment of stringency. Only the washing temperature is varied.

Probe. The nucleic acid probe must be of high specific activity and greater than 50 bp in length so that it can form stable hybrids.

Anticipated Results

After washing the filters the background should be barely detectable with a Geiger counter.

With a high-specific-activity probe $>5 \times 10^7$ cpm/ μ g and an overnight hybridization reaction with a 1-kb unique sequence probe, hybridizing bacterial colonies or bacteriophage plaques can be visualized after a 1 to 18 hr exposure.

Time Considerations

Generally hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots. However, with probes of increasing complexity longer hybridization times are required. This is preferable to increasing the probe concentration from experiment to experiment.

Autoradiography requires 1 to 18 hr.

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Screening
Recombinant
DNA Libraries

6.3.6

Supplement 2